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Award Number: DAMD17-00-1-0232

TITLE: A Normal Epithelial-Mesenchymal Transition as a Model For Metastatic Onset

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REPORT DATE: March 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2002	Annual Summary (1 Mar 01 - 28 Feb 02)

4. TITLE AND SUBTITLE
A Normal Epithelial-Mesenchymal Transition as a Model For Metastatic Onset

5. FUNDING NUMBERS
DAMD17-00-1-0232

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Reports contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Genes and signaling pathways implicated in EMT and the invasiveness of breast cancers include FGF, Notch and T-box and Ets family transcription factors. One goal of this research was to examine the relationship between Notch signaling and the Brachyury T-box transcription factor. Through cloning and characterization of this gene it was shown that Brachyury is not a target of Notch signaling and does not function in mesoderm formation, and therefore EMT. A second T-box family member implicated in breast cancer progression, Tbx2/3, was cloned and characterized. A polyclonal antibody was generated and functional assays performed to determine the role of Tbx2/3 during development and to link these observations to the tumorigenesis observed in breast cancer patients with amplifications of chromosome region 17q23 containing the Tbx2 locus. FGF signaling was also further examined during this funding period but the results of these studies were inconclusive. Functional characterization of Ets factors and subtractive screens aimed at identifying genes regulated by Ets and Tbx2/3 were initiated through a collaborative effort. This effort involves the generation of cDNA macroarrays to facilitate the easy identification and cloning of such genes after subtraction.

14. SUBJECT TERMS
epithelial-mesenchymal transition, metastasis, adhesion, Notch, FGF, Tbx2, Brachyury, Ets, subtractive screen, macroarray

15. NUMBER OF PAGES
33

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

Epithelial-mesenchymal transitions (EMTs) are indicative of cancer metastasis and correlate with poor patient survival. These transitions, however, are a normal part of animal development. The EMT that occurs during mesenchyme formation in the sea urchin is highly representative of the transition that occurs during cancer metastasis in that identical signaling pathways and effector molecules are involved. This transition has been well characterized in the sea urchin and thus provides an *in vivo* model system to further elucidate the changes that cells undergo during EMTs. This study sought to identify signaling pathways and molecular correlates to EMT. In particular studies include; examination of Notch and Fibroblast Growth Factor (FGF) signaling pathways, members of the T-box and Ets transcription factor families and proteins directly involved in the transition such as the Rac, Rho and cdc42 small G-proteins. The goal of these studies is two-fold; first to enhance the knowledge on endogenous EMTs in order to have a basis of comparison for those occurring during metastasis and second, to identify new genes involved in the process and therefore provide potential targets for new therapies aimed at blocking cancer progression.

Body:

During this annual period, significant progress was made on the research proposed. This progress is summarized below.

AIM 1: 'What role do Ets factors play in epithelial-mesenchymal transitions'

Ets proteins comprise a family of winged-helix transcription factors that bind to DNA monomerically at a consensus 10bp site. These factors are regulated by ras dependent MAP kinase phosphorylation of their N-terminal regulatory domains and cooperate with other ras responsive AP1 transcription factors to regulate gene expression (Yang et al., 1996; Wasylyk et al., 1997). Ras has been implicated in oncogenesis since a high number of human cancers exhibit permanently active ras molecules (Downward, 1997). Ets factors, being downstream of ras signaling, may therefore be involved in cancer progression by contributing to the transforming potential of activated ras. Additionally, Ets factors have been directly implicated in breast cancer progression and the invasiveness of certain cancers. Ets proteins overexpressed in mammary tumor cells include ESX/ESE-1, PEA3 and ERM (Dittmer and Nordheim, 1998). Invasive breast cancers produce Ets1 while non-invasive ones do not (Delannoy-Courdent et al., 1996). As well, blockage of Ets dependent transcription by either antisense Ets1 or expression of the Ets DNA binding domain alone is sufficient to block invasiveness *in vitro* (Iwasaka et al., 1996; Chen et al., 1997, Foos et al., 1998). Ets factors have been identified in the urchin (Wei et al., 1999; Qi et al., 1992; Chen et al., 1988) and one such factor has been localized to the ingressing mesenchyme cells (Kurokawa et al., 1999). Overexpression of this factor results in embryos producing an excess of cells, likely mesenchyme, while expression of a C-terminal DNA binding domain alone exhibits the opposite phenotype.
The goal of this aim was then to perturb Ets function in a similar fashion in order to characterize the effects on integrins, cadherins and known effectors of EMT and then to perform a subtractive screen to look for novel genes up or downregulated by Ets factors and therefore possibly EMT.

During this period, characterization of Ets overexpressing embryos indicated that indeed, the resulting excess of cells are mesenchymal in character and do not express the cadherin tumor suppressor. Thus, Ets appears to effect gene expression proximal to EMT, ie. the decrease in adhesion necessary for the transition. Rather than focusing on cataloguing known genes that are Ets targets in the urchin, the goal here is to identify and characterize novel targets. To that end, a collaboration has been entered with the laboratory of Eric Davidson at the California Institute of Technology to generate a series of cDNA macroarrays that can be screened with probes derived from subtractive hybridizations. Arrayed libraries are more advantageous than the standard technologies for such screens as they allow for full genome assay, reproducibility, and easy cloning of differentially regulated genes. Success with this technology and detailed protocols are available (Rast et al., 2000) and the equipment necessary for generation of the array have been supplied. Arrays are currently being generated and should be ready within the next three weeks for screening. The Davidson lab has been very successful in identifying differentially regulated genes using such arrays (see Davidson et al., 2002) hence the choice to proceed in this fashion. mRNA populations are being prepared for subtraction and hybridization to the arrayed filters. These subtractions are focused on identifying genes upregulated or downregulated by Ets factors through subtraction of 'normal' mRNA from Ets overexpressing mRNA. Work during this final

funding period continues to be focused on the identification of differentially expressed genes and ultimately, their characterization during EMT.

AIM 2: 'To characterize the role of FGF and Notch signaling during epithelial-mesenchymal transitions'

Months 1-18 in the 'Statement of Work (SOW) were allocated to study Notch signaling, T-box genes, and the relation between the two. Murine mammary tumor virus (MMTV) induced tumorigenesis previously identified the INT3 gene as a locus for viral insertion (Gallahan and Callahan 1987, 1997). The INT3 gene incodes a mammalian Notch homologue, Notch4. Viral insertion generates a truncated protein consisting of only the intracellular portion of the receptor, and thus, a constitutively active signal. In the sea urchin, a similar form of the Notch protein (LvN^{act}) when expressed results in an increase in the number of migratory mesenchyme cells (Sherwood and McClay 1999).

The first aspect of this specific aim was to characterize Brachyury in the sea urchin as a possible target of Notch signaling as implicated by previous studies (Corbo et al. 1998). A sea urchin homologue of Brachyury was cloned and its expression characterized by Northern blot and by generation of polyclonal antisera against recombinant protein. Brachyury was found to not localize to mesoderm tissue as was suggested by *in situ* observations by another laboratory (Harada et al. 1995). Further, a dominant interfering construct was generated by fusing the DNA binding domain of Brachyury to the repression domain of the *Drosophila* engrailed gene. This construct when expressed in sea urchin eggs had no effect on mesenchyme formation, rather, it blocked the movements of the endoderm during gastrulation. The endoderm tissue was unable to invaginate into the blastocoel and form the embryonic gut. Brachyury, however, did exert a downstream effect non-autonomously indicating that perhaps genes downstream of Brachyury may function in the mesoderm as results from another lab suggest (Davidson et al., 2002). Thus, from this study, Brachyury was determined to not be a target of Notch signaling, nor to be involved in mesoderm formation and therefore not a useful target for therapeutics aimed at blocking EMT. The work, however, is significant in that it shows Brachyury functions in gastrulation movements of the endoderm. Significant adhesive and cytoskeletal changes are necessary for such movements to occur and therefore Brachyury may be important in coordinating these changes.

This work was published within the current period: [Gross, J. M. and McClay, D. R. (2001). The role of Brachyury (T) during gastrulation movements in the sea urchin Lytechinus variegatus. *Dev Biol* **239**, 132-47] and a reprint is attached as an Appendix for more detailed analysis of the results described above.

The next aspect of this specific aim was to identify and characterize other T-box genes in the sea urchin. In particular, the function of Tbx2 was of interest to this study. Recently, Tbx2 has been implicated in breast cancer progression. In breast cancers, amplification of the chromosomal region 17q22-q24 is often observed. Tbx2 is found within this region and is overexpressed in breast cancer cell lines (Barlund et al. 2000). As well, Tbx2 is implicated in breast cancer progression as it was independently identified in a screen of genes possessing the ability to confer immortality to fibroblast cells. Subsequently, Tbx2 was found to indeed be overexpressed

in a subset of primary human breast cancers (Jacobs et al. 2000) and to function in the repression of $p14^{ARF}$, a gene involved in promoting cellular senescence (the inhibition of cellular proliferation and therefore the prevention of 'immortality' or tumorigenesis).

While several T-box transcription factors were cloned during the previous annual period (March 2000 to Feb 2001), during this annual period focus was placed on studies of the Tbx2/3 subfamily of T-box genes based on the above links with breast cancer. A sea urchin homologue of Tbx2/3 was cloned (most genes in vertebrates have been duplicated and therefore in the urchin, the Tbx2/3 subfamily of T-box genes is represented by only one gene – a predecessor of the duplication event that generated individual Tbx2 and Tbx3 genes. Therefore we refer to the sea urchin homologues of Tbx2 and Tbx3 as Tbx2/3 in this study). Tbx2/3 expression was characterized by Northern blot and polyclonal antisera generated against recombinant protein. Tbx2/3 is expressed solely in the nuclei of all dorsal territories of the embryo (ie in all germ layers; Figure 1). Two lines of research were then performed to look at Tbx2/3 function and possible relevance to breast cancer progression; examination of the upstream events necessary for proper Tbx2/3 expression and the downstream effects of perturbing Tbx2/3 function *in vivo*.

The molecular linkage of dorsal specific Tbx2/3 expression within all three germ layers was demonstrated through perturbing the events thought to be involved in the patterning this axis (Figure 2). Tbx2/3 expression is dependent on β-catenin or β-catenin downstream genes as shown through expression of the cytoplasmic tail of cadherin and thereby preventing β-catenin nuclear localization. Tbx2/3 expression is also prevented by ventralization with $NiCl_2$, overexpression of BMP2/4 (Bone Morphogenetic Protein) and through the disruption of the extracellular matrix, and thereby growth factor presentation/signaling. Thus, *in vivo*, Tbx2/3 expression is dependent on a variety of factors, some of which are know to be involved in the ontogeny/progression of breast cancer.

Perturbation of Tbx2/3 function *in vivo* was achieved through overexpression and expression of a hybrid construct encoding a dominant-active form of the protein (Tbx2/3-VP16) as Tbx2 and Tbx3 are thought to be transcriptional repressors (Carreira et al., 1998; He et al., 1999). It appears that expression of these constructs results in complementary phenotypic changes, Tbx2/3 likely dorsalizes the embryo while Tbx2/3-VP16 ventralizes the embryo. Marker costaining of such embryos is currently underway to verify this. While there are no studies in the sea urchin that have looked at cellular proliferation late into development, it is widely accepted that the dorsal territories, which give rise to most of the squamous epithelia that covers the embryo, grow throughout it's lifespan. As described above, studies implicate Tbx2 in breast cancer progression through its repression of $p14^{ARF}$ and thereby preventing cellular senesence. It is also of interest to note that a human pathology, Ulnar-Mammary Syndrome (UMS) resulting from haploinsufficiency of Tbx3 is manifest by hypoplasia of breast tissue, among other defects. It will be of great interest to characterize Tbx2/3 targets in this system to elucidate how these factors function, and what their targets are. To facilitate that, a similar subtractive screen as described above for Ets will be employed to identify Tbx2/3 targets during the remaining funding period. Such targets will then be characterized to ascertain their roles in normal development, through which one might begin to understand the acquisition by breast cancers of a metastic phenotype through unchecked Tbx2 activity. It is out hope that some targets, once

verified in mammalian systems, might then provide novel targets for therapies aimed at preventing this progression. (This work is currently being prepared for submission.)

Months 18-36 in the SOW were allocated to the study of FGF signaling. Work on FGF signaling during this current period also progressed, although negatively. An emphasis was placed on isolation of endogenous sea urchin FGF receptors for characterization and functional assays. Through a combination of degenerate PCR and cDNA library screening with orthologous genes from other organisms, such receptors were sought to no avail. Several primer sets and hybridization conditions were employed but no additional FGF receptors could be identified (one has been described previously, McCoon et al., 1996 and is localized late in developmental to adult muscles). While confidence can be placed in the cloning abilities employed by this author (7 novel sea urchin genes have been cloned under current funding), many months of cloning have proven unsuccessful in identifying novel FGF receptors in the sea urchin. Therefore, injection of a dominant negative FGF1 receptor mRNA from *Xenopus* was employed to assay FGF signaling in the sea urchin and its role in EMT. Such heterologous constructs do indeed function as the endogenous proteins when expressed in the sea urchin embryo (Wikramanayake et al., 1998) so this avenue was pursued. No developmental defects were observed, nor was a block to EMT as hypothesized. This negative result, however, does not conclusively indicate that FGF does not function in the normal EMT of the mesoderm as the possibility exists that a *Xenopus* construct might not recapitulate endogenous FGF signaling in the urchin. While it is still of great interest to characterize FGF signaling in the urchin and to determine the relationship between it and the mesodermal EMT observed during development, until an endogenous FGF receptor can be identified, these experiments are not possible. To that end, the cDNA macroarrays will be screened for FGF receptors using standard screening protocols. The hope is that through a representation of the full genome on several filters, more clones can be assayed and a receptor identified.

EMT has been extensively studied in *in vitro* models where epithelia in culture can be induced to undergo EMT after treatment with various cytokines (Aaronson 1991). Cells necessarily must abrogate these cell-cell interactions in order to gain the ability to migrate from the plane of an epithelium. *In vitro* systems are informative but not truly indicative of what is occurring *in vivo* during either normal development or in a pathological context. The goal of the studies described in this proposal was to characterize an *in vivo* model of EMT in order to extend studies, by comparison, to metastasis and therefore to cancer spread. To that end a series of experiments aimed at looking at potential effectors of EMT rather than solely inductive signals were initiated. During the previous annual period, sea urchin homologues of the Rho GTPases Rho, Rac and cdc42 were cloned. These proteins are intimately associated with the remodeling of adhesive junctions and the actin cytoskeleton (Braga 2000, Schmitz et al. 2000). Rho GTPases are necessary for the formation and disassembly of cell-cell contacts *in vitro* (reviewed in Schmitz et al. 2000). Experiments were designed to ascertain the functions of these GTPases during *in vivo* EMT in the sea urchin embryo. These studies aim to corroborate the *in vitro* observations and perhaps provide a model system in which potential therapies derived to prevent EMT can be tested. Dominant negative and constitutively active forms of each of the sea urchin homologues have been constructed and are currently being expressed in the embryo to assay their function during EMT. These experiments were described in the past annual report but were not in the original SOW. As these were not in the original SOW, and therefore not covered under the

funding of this grant, they have been taken over by another member of the laboratory who will seek independent funding for the study.

Key Research Accomplishments:

- Characterization of the effect of Ets on cadherin expression during EMT and mesoderm specification
- Through a collaborative effort, the generation of cDNA macroarrays to facilitate subtractive screens to search for genes regulated by Ets and Tbx2/3
- Brachyury function characterized through expression of a dominant interfering construct
- Determination that Brachyury is not a target of Notch signaling and is not involved in mesoderm formation and therefore not an aspect of EMT. Brachyury may however, play a role in the mesoderm through activation of non-autonomous signals that then function in mesoderm formation and patterning.
- Characterization of the spatial and temporal aspects of Tbx2/3 expression with polyclonal antisera and northern hybridization
- Characterization of upstream events necessary for Tbx2/3 expression
- Characterization of Tbx2/3 function during development

Reportable Outcomes:

- Gross, J. M. and McClay, D. R. (2001). The role of Brachyury (T) during gastrulation movements in the sea urchin Lytechinus variegatus. *Dev Biol* **239**, 132-47
- Invited Seminar – March 2001, 'Comparative Developmental Biology' Ischia, Italy
- Poster Presentation – July 2001, 'Society For Developmental Biology Annual Meeting' Seattle, WA
- Invited Seminar – October 2001, Department of Biological Sciences – University of Maryland – Baltimore County
- Postdoctoral position in the laboratory of Dr. John Dowling, Harvard University, upon PhD completion

Conclusions:

The overall goal of this research is to provide an *in vivo* insight into the mechanism whereby adhesions are assembled and disassembled during EMT and metastasis and thereby identify potential targets for therapeutics aimed at preventing the spread of breast cancers. Genes and signaling pathways implicated in EMT and the invasiveness of breast cancers include FGF, Notch and T-box and Ets family transcription factors. During the past funding period, studies were performed relating to each of these pathways and factors. Ets factors were shown to increase the amount of mesenchyme in the embryo, in agreement with previous reports. This increase resulted in a decrease in the amount of cadherin in the embryo, and possibly thereby, the acquisition of a mesenchymal phenotype by normally epithelial cells. cDNA macroarrays are being generated to facilitate subtractive screens aimed at identifying novel genes up or downregulated by Ets expression and possibly thereby, EMT and metastasis. A second goal of the study was to examine the relationship between Notch signaling and the Brachyury T-box transcription factor. Through cloning and characterization of this gene it was shown that Brachyury is not a target of Notch signaling and does not function in mesoderm formation, and therefore EMT, rather functioning in the gastrulation movements of the endoderm. A second T-box family member implicated in breast cancer progression, Tbx2/3, was cloned from the sea urchin and characterized. Tbx2/3 is expressed in all dorsal regions of the embryo, downstream of β-catenin or β-catenin downstream genes, BMP2/4 and likely growth factor signaling. Tbx2/3 functions in these regions in pattern formation during embryogenesis, and potentially in cell proliferation. Lastly, FGF signaling was studied. No endogenous FGF receptors could be identified in the urchin so heterologous constructs from *Xenopus* were expressed with no phenotypic manifestations.

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Figure 1

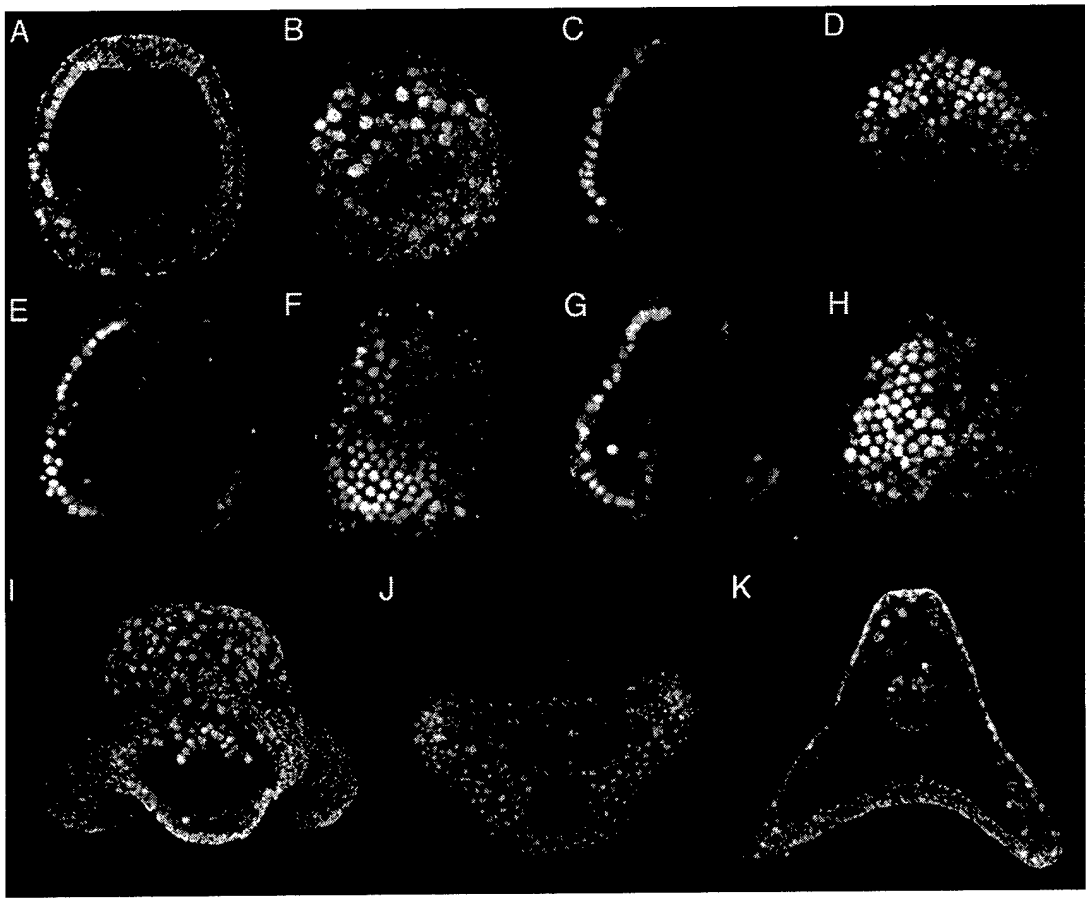

Figure 2

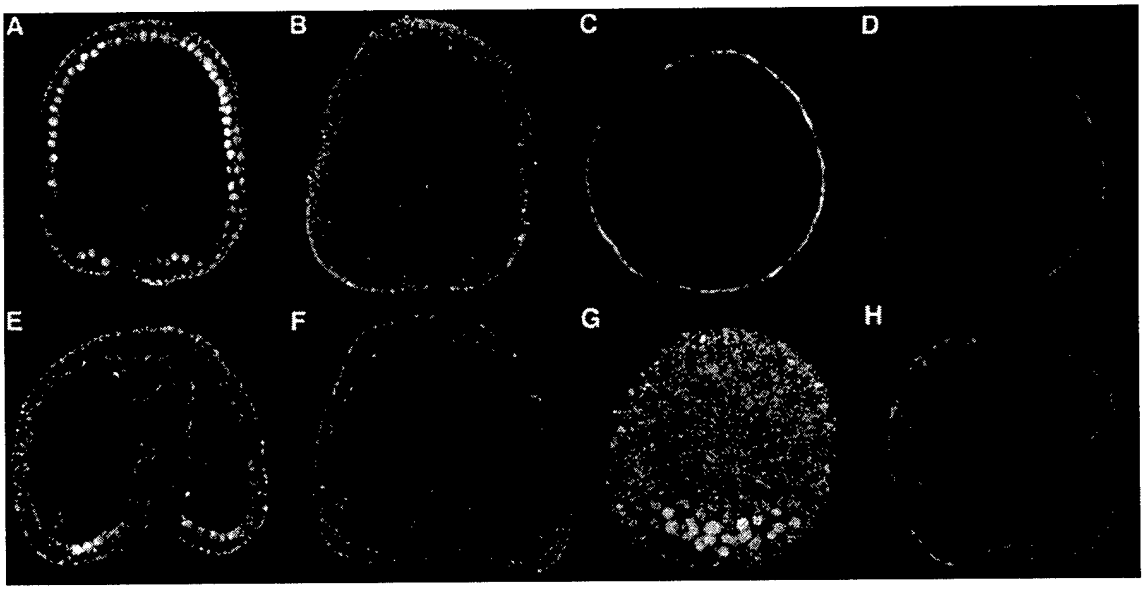

Figure 1: Tbx2/3 is expressed in the nucleus and displays an asymmetric expression pattern during early sea urchin development. (A, C, E, G) Cross sections and (B, D, F, H) surface projections. (A, B) Tbx2/3 expression first appears at mesenchyme blastula stage asymmetrically in the presumptive endoderm and ectoderm as viewed in cross section (A) and in a vegetal surface view (B). (C, D) Early gastrula stage embryos in cross section (C) and a vegetal view (D) maintain asymmetric expression of Tbx2/3 in the presumptive endoderm and ectoderm while the endoderm and mesoderm that have invaginated into the blastocoel do not express protein. (E, F) Mid-gastrula stage embryo (cross section and surface projection of the same embryo). Tbx2/3 expression is maintained asymmetrically in the presumptive endoderm and ectoderm and not in invaginated endoderm or mesoderm. (G, H) Late gastrula expression of Tbx2/3 (cross section and surface projection of the same embryo). Asymmetric expression is observed in the endoderm at this stage and in the ectoderm. Expression is also observed at this stage in all of the skeletogenic mesenchyme cells. (I-K) Late expression of Tbx2/3. (I) Animal view of early pluteus embryo optically sectioned to remove the most superficial layers of ectoderm exposing the archenteron and stomodaeum. Expression is observed asymmetrically in the surface ectoderm and the length of the archenteron. (J) Vegetal surface view of a pluteus stage embryo. Expression of Tbx2/3 is observed asymmetrically in all ectoderm cells up to the ciliated band that separates oral and aboral territories. Expression is also observed in the distal most nuclie of the extending pluteus arms. (K) Vegetal cross section of a late pluteus embryo. Asymmetric expression is maintained in cells of the ectoderm, endoderm and skeletogenic mesoderm.

Figure 2: Perturbations to embryonic patterning affect Tbx2/3 expression. $NiCl_2$ ventralizes sea urchin embryos. Ventral LvBrac expression expands to all ectoderm cells (A) while Tbx2/3 is not expressed in these embryos (B). (C, D) Injection of ΔLvG-cadherin mRNA animalizes the embryo through binding to endogenous β-catenin and preventing its nuclear localization. These embryos lack endoderm and mesoderm and express the ventral specific EctoV antigen uniformly (C). They do not, however, express any Tbx2/3 protein (D). (E, F) Overexpression of BMP2/4 radializes the ectoderm of the embryo as indicated by the formation of multiple tri-radiate spicules (Angerer et al., 2000). Such embryos express normal levels of vegetal Brac around the blastopore but do not express ventral Brac (E) or dorsal Tbx2/3 (F) indicating that overexpression of BMP2/4 antagonizes normal dorsal/ventral axis specification events. (G, H) Disruption of the extracellular matrix with βAPN, a drug that prevents collagen crosslinking and potentially thereby, growth factor signaling/presentation. Ventral Brac is not expressed while vegetal expression is present in these embryos (G). (H) Tbx2/3 is also not expressed under such conditions indicating that an intact ECM is necessary for polarity about the dorsal/ventral axis and the expression of both dorsal and ventral markers.

Developmental Biology 239, 132–147 (2001)
doi:10.1006/dbio.2001.0426, available online at http://www.idealibrary.com on IDEAL®

The Role of Brachyury (T) during Gastrulation Movements in the Sea Urchin *Lytechinus variegatus*

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The studies described here sought to identify and characterize genes involved in the gastrulation and morphogenetic movements that occur during sea urchin embryogenesis. An orthologue of the T-box family transcription factor, *Brachyury*, was cloned through a candidate gene approach. *Brachyury* (*T*) is the founding member of this T-box transcription factor family and has been implicated in gastrulation movements in *Xenopus*, zebrafish, and mouse embryogenesis. Polyclonal serum was generated to LvBrac in order to characterize protein expression. LvBrac initially appears at mesenchyme blastula stage in two distinct regions with embryonic expression perduring until pluteus stage. Vegetally, LvBrac expression is in endoderm and lies circumferentially around the blastopore. This torus-shaped area of LvBrac expression remains constant in size as endoderm cells express LvBrac upon moving into that circumference and cease LvBrac expression as they leave the circumference. Vegetal expression remains around the anus through pluteus stage. The second domain of LvBrac expression first appears broadly in the oral ectoderm at mesenchyme blastula stage and at later embryonic stages is refined to just the stomodael opening. Vegetal LvBrac expression depends on autonomous β-catenin signaling in macromeres and does not require micromere or veg2-inductive signals. It was then determined that LvBrac is necessary for the morphogenetic movements occurring in both expression regions. A dominant-interfering construct was generated by fusing the DNA binding domain of LvBrac to the transcriptional repression module of the *Drosophila* Engrailed gene in order to perturb gene function. Microinjection of mRNA encoding this LvBrac-EN construct resulted in a block in gastrulation movements but not expression of endoderm and mesoderm marker genes. Furthermore, injection of LvBrac-EN into one of two blastomeres resulted in normal gastrulation movements of tissues derived from the injected blastomere, indicating that LvBrac downstream function may be nonautonomous during sea urchin gastrulation. © 2001 Academic Press

Key Words: Brachyury (T); sea urchin; gastrulation; morphogenesis.

INTRODUCTION

The molecular and cellular basis of morphogenesis remains one of the least understood areas of developmental biology. While the accumulation of data regarding the signaling networks employed by cells and tissues has increased exponentially over the last half century, little progress has been made in understanding how these cells and tissues move and behave such that a properly formed embryo results (see Fraser and Harland, 2000). Two of the best understood processes of embryonic morphogenesis are those of gastrulation in *Xenopus* (Winklbauer and Keller, 1996; Wacker *et al.*, 1998; Winklbauer and Schurfeld, 1999) and gastrulation in the sea urchin (Hardin, 1996; Ettensohn, 1999; Wessel and Wikramanayake, 1999). The cell movements and shape changes in these organisms have been well described such that a coherent understanding of the behaviors associated with gastrulation is emerging. The breadth of descriptive information in these organisms makes them ideal models for a molecular dissection of the process (ex., Wallingford *et al.*, 2000; Djiane *et al.*, 2000; Tada and Smith, 2000).

The dynamics of gastrulation and the molecular components underlying it are easily studied in the sea urchin with its well-defined lineages, optical clarity, and amenability to surgical and molecular perturbation. A wealth of knowledge exists encompassing movements and behaviors during

The nucleotide sequence reported in this paper will appear in the GenBank database with the Accession No. AF298811.

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0012-1606/01 $35.00

sea urchin gastrulation both from the classic embryologists (Gustafson and Wolpert, 1963, 1967; Hortsadius, 1973) and contemporaries (McClay, 1991; Davidson, 1993; Hardin, 1996; Ettensohn, 1999).

Invagination of the archenteron occurs through a well-described sequence of events. First, a ring of cells in the center of the vegetal plate become wedge-shaped with constricted apices and bulbous basal sides (Nakajima and Burke, 1996). These "bottle cells" likely are presumptive secondary mesenchyme cells (SMCs) based on their location in the plate when compared to the fate map of this region (Ruffins and Ettensohn, 1996). Kimberly and Hardin (1998) provided some evidence as to their possible function by laser ablating 90–180° arcs of bottle cells and observing the result on primary invagination. Invagination was retarded in the areas surrounding the ablated cells while those surrounding unablated bottle cells invaginated normally. The next phase of archenteron formation involves convergent extension during which cells of the gut rearrange and interdigitate, thereby elongating the gut tube approximately two-thirds the distance across the blastocoel (Hardin and Cheng, 1986; Hardin, 1989). This leads to the last phase of gastrulation during which the SMCs at the tip of the archenteron pull it into contact with the overlying ectoderm (Hardin, 1988; Hardin and McClay, 1990). Additionally, as the archenteron is involuting and undergoing convergent extension during the secondary and tertiary phases of invagination, more cells are added to the base of the gut as evinced by fate mapping of the veg1 and an2 cell layers (Logan and McClay, 1997; Martins *et al.*, 1998; Ransick and Davidson, 1998).

As part of an ongoing effort to elucidate these events at a molecular level, a candidate gene approach was employed to isolate and characterize genes hypothesized to be involved in the process. A *Lytechinus variegatus* orthologue of *Brachyury* (*T*) was cloned and characterized as such a candidate. *Brachyury* is the founding member of the T-box family of transcription factors which has been identified in numerous and diverse animal species (reviewed in Smith, 1999; Papaioannou and Silver, 1998). Originally identified in the mouse over 70 years ago (Dobrovolskaia-Zavadskaia, 1927), the gene was positionally cloned in 1990 by Herrmann and colleagues (Herrmann *et al.*, 1990). Subsequent identification of a fly gene, *omb*, and multiple mouse T-like genes initiated a field of research on the newly termed T-box family (Herrmann *et al.*, 1990; Pflugfelder *et al.*, 1992; Bollag *et al.*, 1994). *T* was shown to be a transcription factor and to localize to the nucleus in both mouse and zebrafish (Wilkinson *et al.*, 1990; Schulte-Merker *et al.*, 1992; Kispert *et al.*, 1994). Subsequent studies have identified numerous family members comprising different subfamilies of T-box genes in various animals. These genes are quite similar to one another within the T-box (DNA binding) region, but outside of this bear little similarity to each other or other family members.

Studies of Brachyury mutants in the mouse have demonstrated several key features of the phenotype (Wilson *et al.*, 1993, 1995; Wilson and Beddington, 1997). Homozygote T mutant mice die midgestation while hemizygous embryos exhibit a reduction in tail length (Dobrovolskaia-Zavadskaia, 1927). Chimeric analyses of these mice have demonstrated that the tail defects are due to an accumulation of mutant cells at the primitive streak, as they are unable to traverse it and colonize more rostral regions of the embryo (Wilson *et al.*, 1993, 1997). Expression of T in homozygous mutant T/T ES cells rescues this phenotype as cells now traverse the streak and populate rostral tissues. In fact, T/T mutant ES cells expressing higher levels of T than wild-type cells migrate from the streak prematurely and populate only the rostral-most tissues, whereas the wild-type cells colonize both rostral and caudal tissues (Wilson and Beddington, 1997). These studies, in demonstrating a role for the T gene in gastrulation movements, caused us to hypothesize a similar role in the sea urchin. In this paper, we report the cloning and characterization of a *Lytechinus variegatus Brachyury* orthologue (LvBrac). By generating polyclonal sera against LvBrac, we demonstrate that LvBrac is localized in two distinct domains: (1) to the nuclei of a torus of cells around the blastopore and (2) to the oral ectoderm and stomodaeum. We then demonstrate that functional perturbation of LvBrac blocks archenteron invagination from the vegetal plate and therefore the movements of gastrulation. Further, we show that LvBrac might act cell nonautonomously during sea urchin development.

MATERIALS AND METHODS

Animals

Sea Urchins (*Lytechinus variegatus*) were obtained from Susan Decker (Hollywood, FL) or Tracy Andacht and Jennifer Keller (Duke University Marine Laboratory). Gametes were harvested and cultured at 23°C as described by Hardin *et al.* (1992).

Cloning of LvBrachyury

Degenerate primers were designed to the amino acids YIHPDSP (forward)/AVTAYQN (reverse) and used in a PCR of cDNA prepared from midgastrula poly(A)$^+$ mRNA. PCR conditions were 96°C, 60 s; 40°C, 60 s; and 72°C, 2 min 45 s for 45 cycles. The amplified 234-bp product was gel purified, cloned into pGEMT vector (Promega), and sequenced with T7 and SP6 primers (Duke Sequencing Core). Clones were identified as LvBrac PCR products by BLAST search.

Library Screening and Sequence Analysis

A λZAP-II midgastrula cDNA library (Stratagene) was screened by using an amplified PCR product generated from the cloned fragment as a probe. Probe was random-primed labeled with P^{32} by using rediprime II random primed labeling kit (AmershamPharmacia Biotech) and hybridized for 20 h at 42°C in 50% formamide, 5× SSPE, 0.1% SDS, 5× Denhardt's, 100 μg/ml salmon sperm DNA. Filters were washed three times for 5 min at room temperature in 2× SSC, 0.1% SDS and two times for 45 min at 52°C in 1× SSC, 0.1% SDS; dried and placed on Kodak XAR film (Eastmen Kodak Co., Rochester, NY) for 48 h at −70°C with an intensifying screen.

Potential positive plaques were replated and further screened twice with two resulting positive plaques being excised and ligated into the *Eco*RI site of pBS SK− (Stratagene). These clones were bidirectionally sequenced (Duke Sequencing Core) and sequences aligned by using AssemblyLign (International Biotechnologies, Inc.). Phylogenetic analysis was performed by using PAUP.

Northern Blotting

A 1% agarose/formaldehyde gel was employed for Northern blotting. Briefly, 3 μg/lane poly(A)$^+$ mRNA (isolated with Quickprep mRNA purification kit, AmershamPharmacia Biotech) was fractionated for 3.5 h by electrophoresis, transferred onto a Nylon membrane by using the Turboblotter System (Schleicher and Schuell; Keene, NH), and hybridized with a 354-bp fragment of LvBrac corresponding to amino acids 387–503. The blot was prehybridized for 3 h in 50% formamide, 5× Denhardt's, 5× SSPE, 0.5% SDS, 100 μg/ml salmon sperm DNA, then hybridized for 20 h at 42°C in fresh buffer containing the random-primed labeled probe. Washes were two times for 5 min in 6× SSPE, 0.5% SDS at room temperature, one time for 45 min in 1× SSPE, 0.1% SDS at 37°C, and one time for 45 min in 1× SSPE, 0.1% SDS at 50°C. The blot was wrapped in plastic wrap and placed on film for 72 h at −70°C with an intensifying screen. It was then stripped in 50% formamide, 6× SSPE for 30 min at 65°C and reprobed as above with a *Lytechinus pictus* ubiquitin fragment as a loading control.

Antibody Production

A full-length LvBrac fusion protein was expressed by subcloning LvBrac into the pGEX4T-1 expression vector [glutathione *S*-transferase (GST) expression system; AmershamPharmacia Biotech]. Expressed, purified protein (80 μg) was mixed 1:1 with Freund's complete adjuvant and injected into each of three guinea pigs (Charles River, Raleigh, NC). Then, 21, 42, and 70 days later, animals were each boosted with 80 μg protein mixed 1:1 with incomplete Freund's. Bleeds were performed at days 31, 53, and 80 postinjection and serum isolated as described (Harlow and Lane, 1988).

Western Blotting

For Western analysis, polyclonal serum from one guinea pig (Pig H) was purified on an affinity column (AminoLink Immobilization kit; Pierce, Rockford, IL), concentrated (Centricon 30; Amicon Inc., Beverly, MA), and Western analysis performed. Briefly, 1000 midgastrula embryos were homogenized, boiled, and run on a 10% SDS–PAGE gel. Gel was then blotted to nitrocellulose, blocked 4 h at 4°C in 5% milk/TBS, and probed overnight at room temperature with a 1:100 dilution of the affinity-purified α-Brac antibody in 5% milk/TBS. Blot was washed three times with PBS and goat anti-guinea pig HRP-tagged 2° antibody applied for 2 h at room temperature.

Immunolocalization and Image Analysis

Embryos were fixed in 2% paraformaldehyde/60% ASW for 10–12 min at room temperature, then permeablized for 60 s with 100% ice-cold methanol. They were then rehydrated and washed two times with ASW and three times with PBS, blocked for 10–20 min in PBS/4% normal goat serum (GibcoBRL), incubated overnight at 4°C in primary antibody/4% NGS, washed four times in PBS, blocked as above, and incubated for 60 min at room temperature in secondary antibody/4% NGS (Cy3 or Cy5-conjugated; Jackson ImmunoResearch Laboratories). Embryos were then washed four times in PBS and mounted in 70% glycerol. LvBrac serum was diluted 1:1000 for all images. PMC-specific monoclonal antibody (mAB) 1G8, SMC-specific mAB SMC-1 (Sweet *et al.*, 1999), and mouse Bam1 α-Notch pAb (Sherwood and McClay, 1997) were used with the above fixation and incubation conditions as undiluted monoclonal supernatants and 1:1000 dilution, respectively. All images were obtained using a 40× Plan-Neofluar oil immersion objective (NA = 1.3) on a Zeiss laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY) mounted on a Zeiss Axiovert inverted microscope. Where necessary, 1-μm sections from single-label images were rendered into 2D projections using Zeiss confocal software while double-labeled images were taken sequentially using appropriate filters and subsequently overlayed using Adobe Photoshop 5.0. Embryos incubated with preimmune serum did not stain positively at any of the stages examined nor did embryos stained with polyclonal serum preincubated with fusion protein prior to staining (data not shown). All images shown were stained with serum from a single guinea pig (Pig H) but serum from two other guinea pigs (G and I) produced identical results.

Chemical Treatments

Treatment of embryos with $NiCl_2$ was performed as described in Hardin *et al.* (1992) and LiCl as described in Logan *et al.* (1999).

Embryo Manipulations

Embryos to be dissected were transferred by mouth pipet to calcium-free SW. They were then inserted into a Kiehart chamber (Kiehart, 1982) that was inverted relative to the original protocol. A joystick micromanipulator was used to manipulate a glass needle as the dissection tool. The needle was broken to make a blunt tip with an inside diameter just smaller than the diameter of the cells to be removed or transplanted. Micromeres were removed by suction using a Gilmont 2-ml microsyringe containing silicon oil attached to the needle for suction. Micromere/veg2 removals were performed as above except that after micromeres were removed at the 16-cell stage, the embryos were allowed to undergo two rounds of division at which point the veg2 layer was then removed. After surgeries were completed, the chamber was flooded gradually with ASW and the embryos transferred to 96-well plates for culture.

LvBrac-ENgrailed Construct

LvBrac-ENgrailed fusion construct was made by PCR amplifying the region encoding amino acids 2–295 of the *Drosophila* engrailed gene encoding the repression domain from the *siamois–engrailed* pXT7 plasmid (Fan and Sokol, 1997; gift from S. Sokol) with an additional overhang of 20 bp complementary to the 3′ terminus of the LvBrac DNA binding domain at the 5′ end of the fragment and an *Xma*I site at the 3′ terminus. The putative LvBrac DNA binding region corresponding to that mapped in *Xenopus laevis* and *Danio rerio* (amino acids 1–252; Conlon *et al.*, 1996) was PCR amplified with an additional 20 bp complementary to the 5′ terminus of the *engrailed* repression domain at the 3′ end of the fragment and an *Xba*I site at the 5′ terminus. These fragments were then purified, their concentrations determined and used in a second PCR containing 1:100 dilutions of each fragment with the following cycling parameters; 96°C, 60 s; 50°C, 90 s; 72°C, 4 min 30 s for 40 cycles

with the 5′ Brachyury primer and 3′ engrailed primer employed above. A product of 1638 bp was gel extracted, digested sequentially with *Xba*I and *Xma*I, and ligated into dephosphorylated *Xba*I/*Xma*I digested pBS SK−. The construct was sequenced bidirectionally to verify successful LvBrac-ENgrailed fusion (Duke Sequencing Core). An *engrailed*-expressing plasmid (a generous gift from L. A. Angerer; Wei *et al.*, 1999) was employed as an injection control.

mRNA Preparation and Injection

LvBrac-EN was linearized with *Cla*I and EN-pXT7 was linearized with *Bam*HI. These were used as templates to generate *in vitro* transcribed 5′ capped mRNAs by using the T3 (LvBrac-EN) or T7 (EN-pXT7) mMessage mMachine kits (Ambion). Concentrations of mRNAs were estimated by comparison to known amounts of RNA by both gel electrophoresis and dotting onto a 0.6% agarose gel. Eggs were prepared and injected as described (Sherwood and McClay, 1999). LvN^{act} and ΔLvG-cadherin were linearized and injected as described (Sherwood and McClay, 1999; Logan *et al.*, 1999).

RESULTS

Isolation of Lytechinus variegatus Brachyury (LvBrac)

LvBrac was PCR amplified from a midgastrula stage cDNA pool by using degenerate oligonucleotides designed to homologous regions of the *Brachyury* T-box identified in various animal species (primer sites underlined in Fig. 1A). Amplification and sequencing of a 234-bp fragment identified it as a *Lytechinus variegatus Brachyury* orthologue. A midgastrula cDNA library was then screened and two LvBrac cDNA clones recovered, one a full-length and the other an N-terminal fragment. Two other sea urchin orthologues have been previously identified, one in *Hemicentrotus pulcherrimus* (Harada *et al.*, 1995) and the other in *Strongylocentrotus purpuratus* (Peterson *et al.*, 1999).

Deduced Amino Acid Sequence and Phylogenetic Analysis

LvBrac encodes a 503-amino acid protein based on the predicted open reading frame from the primary sequence data (Fig. 1A). Interestingly, the predicted open reading frame of LvBrac is significantly longer than HpTa in that the HpTa protein is 434 amino acids in length (Harada *et al.*, 1995). LvBrac is one amino acid longer than SpBra, with a T inserted at codon 402 of LvBrac (Peterson *et al.*, 1999a). To insure the accuracy of the LvBrac sequence, additional cloning and sequencing was performed. cDNAs were reverse transcribed from mRNA isolated from three independent females (mesenchyme blastula and midgastrula stages) and used in PCRs to amplify the C-terminal region of LvBrac. Sequences of these fragments were identical to that obtained from the cDNA library in Fig. 1A (data not shown). Figure 1B shows a protein alignment of these three sea urchin *Brachyury* orthologues. Figure 1C shows a phylogenetic tree of *Brachyury* genes. The tree supports LvBrac as a member of the *Brachyury* subfamily of T-box genes.

LvBrac Expression and Localization

Northern blotting of LvBrac mRNA (Fig. 2) revealed that a 3.22-kb message first appears at mesenchyme blastula stage and perdures through prism stage, finally declining during the pluteus stage (48 h postfertilization). This temporal mRNA expression correlates well with the onset and duration of gastrulation in *Lytechinus variegatus*. *In situ* analyses of HpTa and SpBra revealed that the message is initially expressed in the vegetal plate of the embryo and later expressed in the SMCs delaminating from the tip of the archenteron (Harada *et al.*, 1995; Peterson *et al.*, 1999). To characterize the temporal and spatial distribution of LvBrac protein, a polyclonal antiserum was generated in guinea pigs against recombinant full-length LvBrac protein. This serum was tested for immunoreactivity via protein analysis on SDS–PAGE gels and immunofluorescent staining of fixed embryos.

A Western blot of midgastrula protein extracts was probed with affinity-purified LvBrac sera to ascertain its specificity (Fig. 3). A single immunoreactive band of approximately 54 kDa was observed on the blot, indicating that the polyclonal sera generated was specific to one protein, identical in size to that predicted for LvBrac. Whole-mount immunoflourescent analysis was next performed to localize LvBrac protein during embryogenesis. LvBrac expression appears in two distinct domains: (1) vegetally around the blastopore and (2) in the oral ectoderm of the animal hemisphere.

Vegetal Expression

Figure 4 shows the vegetal protein expression pattern in mesenchyme blastula (Figs. 4A and 4B), early gastrula (Figs. 4C and 4D), and late gastrula (Figs. 4E and 4F) embryos (cross sections A, C, E and vegetal views B, D, F). As expected, LvBrac protein is localized to the nucleus as reported for the mouse and zebrafish orthologues (Wilkinson *et al.*, 1990; Schulte-Merker *et al.*, 1992). There are several notable points regarding the expression pattern. The protein is expressed in a dynamic fashion in and around the vegetal plate of the embryo in a circumferential or torus-shaped region of presumptive endoderm beginning at the mesenchyme blastula stage (Fig. 4B). Examining a mesenchyme blastula embryo in cross section reveals that the extent of the torus lies beyond the lateral edges of the vegetal plate extending into adjacent, more animal epithelial cells. No staining was observed within the central region of the plate, thus generating the torus shape. Gastrula-stage embryos (Figs. 4C–4F) reveal a similar pattern in that the LvBrac torus is maintained around the vegetal plate of the embryo (Figs. 4D and 4F) while cross sections demonstrate that the lateral extent of protein expression lies in the nuclei of noninvoluted cells adjacent

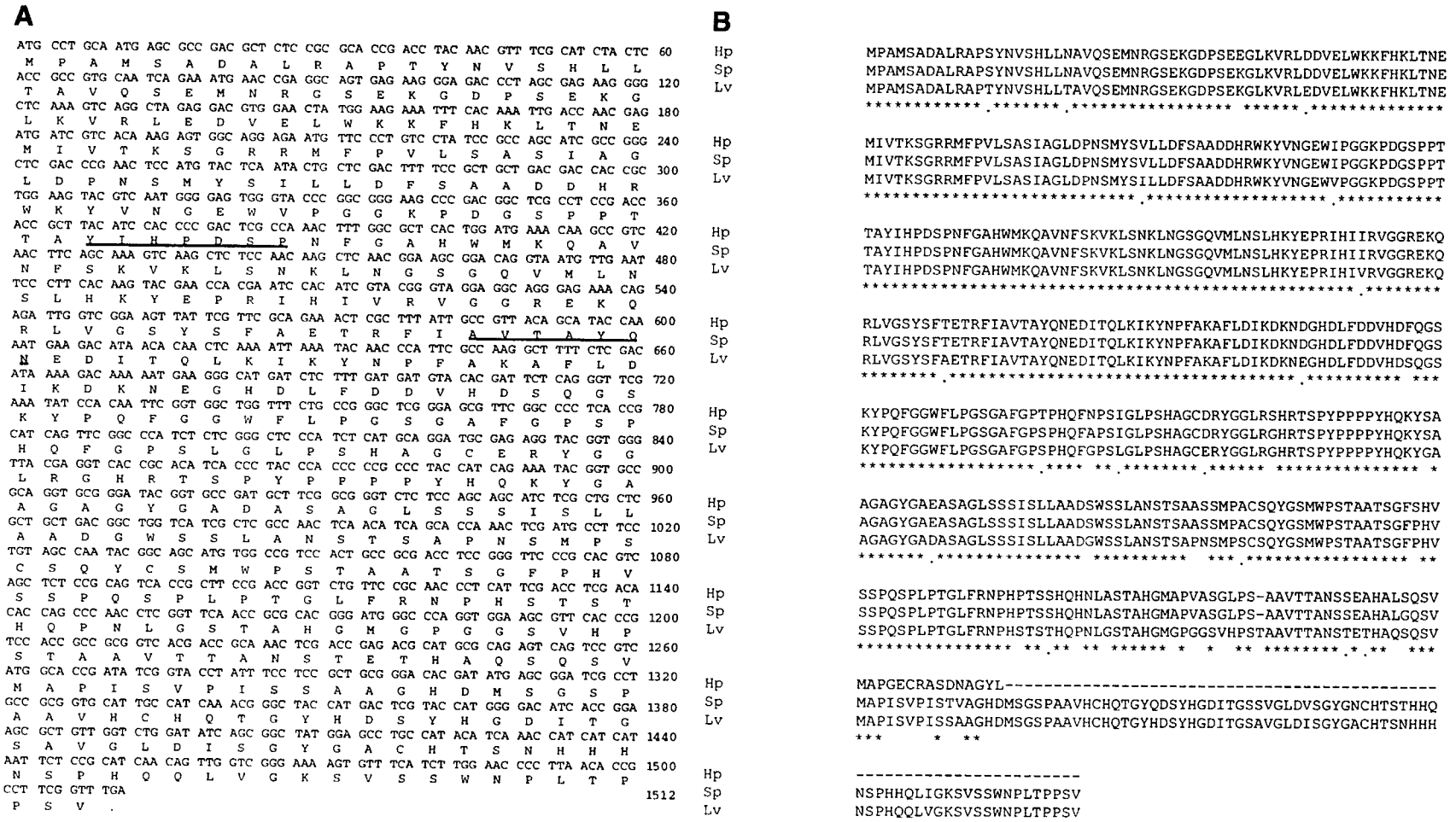

FIG. 1. (A) Nucleotide and predicted amino acid sequences of LvBrac. Sites used for degenerate PCR primers are underlined.(B) Protein alignment of HpTa (Hp, Hemicentrotus pulcherrimus), SpBra (Sp, Strongelocentrotus purpuratus) and LvBrac (Lv, Lytechinus variegatus). Identical residues are denoted by an (*) and conserved residues by a (.). (C) Phylogenetic tree of LvBrac and Brachyury orthologues from other organisms. Bootstrap values indicated on nodes.

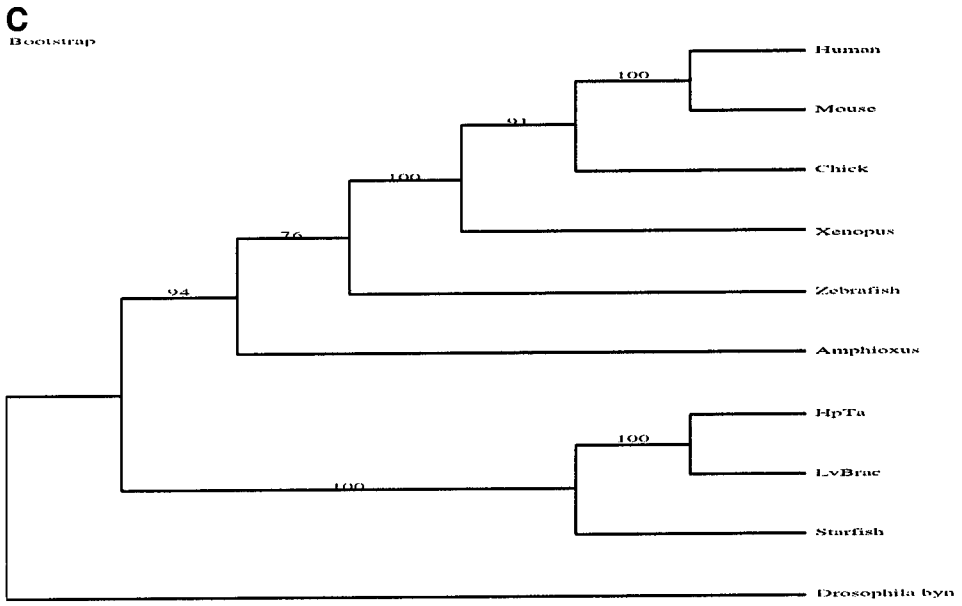

FIG. 1—*Continued*

to those having involuted (Figs. 4C and 4E). Expression is maintained vegetally in the anus of the embryo into pluteus stages (Fig. 5C).

Animal Expression

The second domain of LvBrac expression appears in the animal hemisphere from late mesenchyme blastula stages through gastrula stages in the oral ectoderm. Cross section and surface views of this pattern are illustrated in Fig. 5. Figure 5A is a cross section of a mesenchyme blastula embryo revealing both vegetal expression and expression in a lateral patch of oral ectoderm cells. Viewing the surface of an early gastrula-stage embryo also demonstrates, in addition to the vegetal expression, a similar expression region in the oral ectoderm as observed in cross section (Fig. 5B). Expression initially appears to be a domain of oral ectoderm larger than that which forms the stomodaeum. It appears that this domain, however, is refined prior to pluteus stages where only the stomodaeum expresses LvBrac (Fig. 5C). Interestingly, *Brachyury* orthologues in starfish and hemichordates display similar stomodael mRNA expression patterns of *Brachyury* as that observed here for LvBrac protein (Tagawa *et al.*, 1998; Shoguchi *et al.*, 1999).

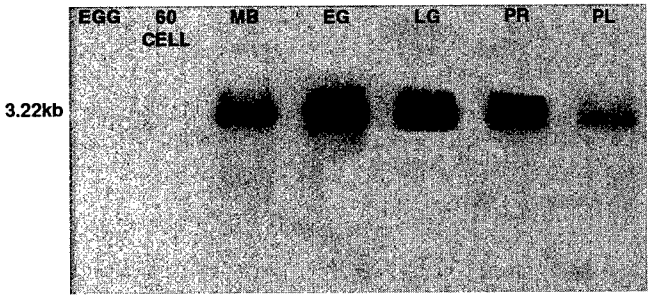

FIG. 2. Developmental Northern blot of LvBrac expression. 3ug/lane of poly(A)$^+$ RNA was loaded as calculated by OD_{260} and loading verified by probing the blot with a *Lytechinus pictus* ubiquitin fragment (data not shown). Egg; 60 cell; MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula; PR, prism; PL, pluteus larva.

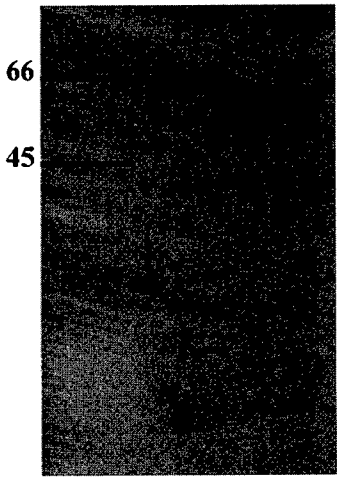

FIG. 3. Western analysis of midgastrula protein extracts (1000 embryos) using affinity purified polyclonal LvBrac serum. A single immunoreactive band of approximately 56 kDa appeared, in agreement with the predicted open reading frame size of LvBrac.

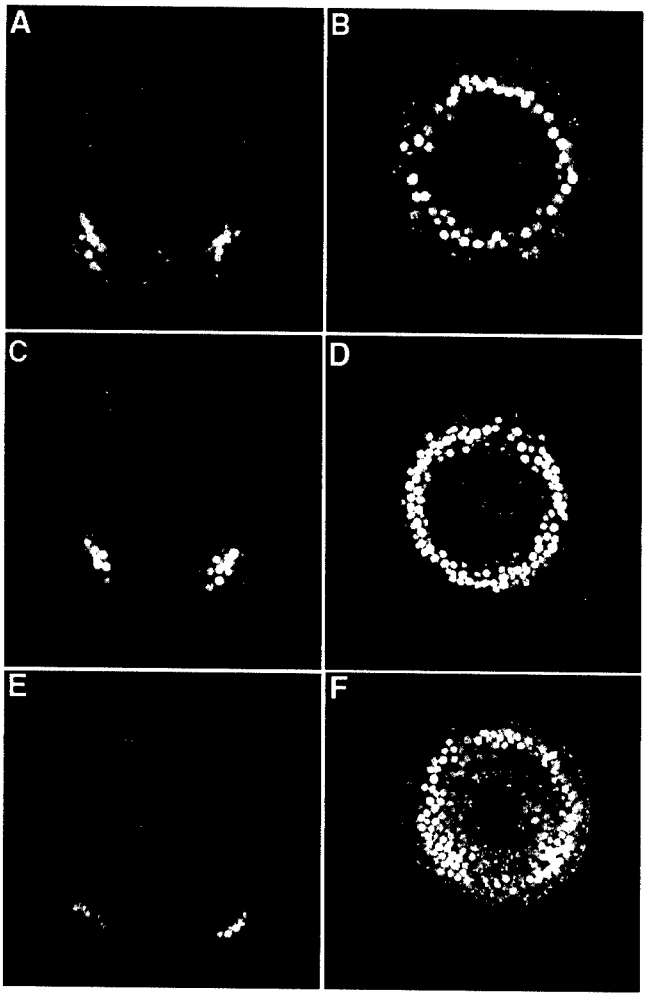

FIG. 4. LvBrac displays a dynamic expression pattern in the vegetal hemisphere during sea urchin gastrulation. (A, C, E) Cross sections and (B, D, F) vegetal views. (A, B) LvBrac first appears at mesenchyme blastula stage in a circumferential region of nuclei within the vegetal plate, (A) Cross section of an embryo where the lateral extent of the expression domain can be viewed. (B) Vegetal view where the full diameter of the expression is shown. (C, D) Slightly older early gastrula embryos where the archenteron is beginning primary invagination. (E, F) Late gastrula embryos where the archenteron has nearly reached the top of the blastocoel and therefore completed gastrulating. In (A-F), the area of expressing cells appears to remain constant as tissue has passed through the blastopore and invaginated into the blastocoel.

Treatment of embryos with $NiCl_2$ results in a disruption of oral–aboral (ventral–dorsal) patterning (Hardin *et al.*, 1992). Embryos perturbed in this manner display defects in ectodermal patterning in that a circumferential stomodaeum forms around the animal rather than at a localized site and ectopic sites for spiculogenesis occur. These animals are radialized and now express oral markers around their entire circumference except the vegetal plate. Embryos treated with 1 mM $NiCl_2$ were stained with LvBrac serum (Fig. 6) at early gastrula stage. Rather than a patch of oral ectoderm expressing LvBrac protein, the entire ectoderm expresses it. Normal vegetal expression is present in these animals.

LvBrac and Endoderm Identity

Brachyury has long been studied in vertebrates for its role in mesoderm formation (reviewed in Herrmann, 1995) and has been implicated for SMC identity in the sea urchin embryo (Harada *et al.*, 1995; Peterson *et al.*, 1999). Figure 7A shows the colocalization of LvBrac and the PMC marker, 1g8. Nuclear LvBrac staining is not present in PMCs. Figures 7B–7E show colocalization of LvBrac and LvNotch. At the mesenchyme blastula stage and through gastrula stages, LvNotch is expressed in a characteristic pattern that delineates endoderm from mesoderm. LvNotch is expressed along the apical side of the endoderm and is markedly downregulated in the prospective secondary mesenchyme (Sherwood and McClay, 1997, 1999, 2001). Using LvNotch as a marker of the endoderm–mesoderm boundary, Fig. 7 demonstrates that LvBrac is expressed exclusively in the endoderm of the vegetal plate and is not present in the prospective SMCs: the area where the LvNotch protein has been internalized (mesenchyme blastula, Figs. 7A and 7B) or delaminating SMCs (early and late gastrula, Figs. 7D and 7E). Overexpression of mRNA encoding an activated form of the LvNotch receptor results in an increase in SMCs at the expense of endoderm (Sherwood and McClay, 1999). LvBrac expression was not observed in the ectopic SMCs induced from injection of LvN^{act} mRNA (1.25 pg/pl, animal view of ectopic SMCs resulting from LvN^{act} mRNA injection, Fig. 7F).

A vegetal plate fate map of the mesenchyme blastula stage embryo has been reported (Ruffins and Ettensohn, 1996) and in this study it was noted that the vegetal plate contains 155 endodermal precursor cells lying circumferentially around the prospective secondary mesenchyme at the center of the plate. The number of LvBrac-positive nuclei observed in mesenchyme blastula-stage embryos was 80 ± 14 (n = 10). It is therefore likely that the early vegetal expression of LvBrac is veg2-derived. As gastrulation proceeds, expression then spreads to veg1-derived endoderm as late expression is observed in the anus, a veg1 derivative. The position of DiI-labeled veg1 cells at mesenchyme blastula stage relative to the pattern of LvBrac expression at this stage reinforces this conclusion (data not shown).

LvBrac As a Marker of Invagination

The expression pattern of LvBrac indicates that the protein is present in regions of the embryo that are actively undergoing morphogenesis, namely, the blastopore and the oral ectoderm. Structures formed from these two regions employ similar morphogenetic behaviors of invagination.

The circumferential expression pattern of LvBrac around the involuting archenteron is quite striking. As gastrulation

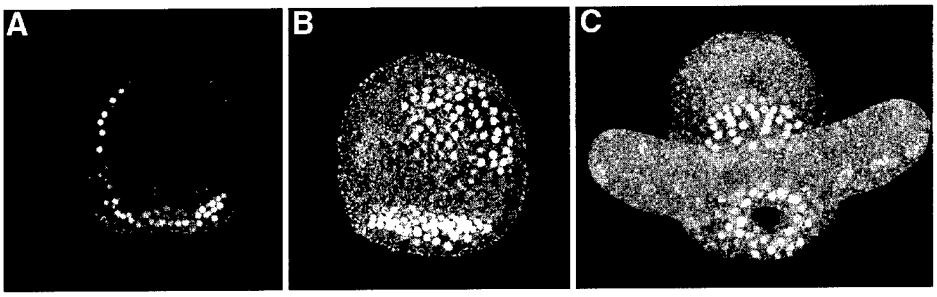

FIG. 5. Oral expression of LvBrac during mesenchyme blastula, early gastrula and pluteus stages. (A) Cross section of a mesenchyme blastula embryo indicates that LvBrac protein is present in the ectoderm of the embryo. (B) Surface view of LvBrac expression in an early gastrula embryo. Note that expression is present in distinct animal and vegetal domains. (C) Expression of LvBrac in early pluteus embryo. Expression is maintained in both the oral ectoderm that will form the stomodaeum and vegetally in the anus.

proceeds and the archenteron involutes progressively further into the embryo, LvBrac expression remains localized to a rather constant region lateral to the blastopore opening. As the endoderm cells traverse into the blastocoel, protein disappears from the nuclei of involuted cells, while expression appears in nuclei of adjacent, noninvoluted cells. Thus, expression of LvBrac is very dynamic in that only cells at the lip of the blastopore express the protein. This observation is further supported in embryos doubly stained with LvBrac and LvNotch (Figs. 7B–7E). By using the LvNotch receptor's apical expression as a marker for endoderm invagination and comparing that pattern of expression to the expression of LvBrac around the blastopore, this observation is reinforced as cells involute (as marked by apical LvNotch) and they no longer express LvBrac in their nuclei. Thus, endoderm cells express LvBrac, involute, and subsequently no longer express the protein.

Autonomy of LvBrac Expression—A Downstream Target of Nuclear β-Catenin?

It has been demonstrated that the micromeres induce mesodermal specification through the Notch receptor (Sherwood and McClay, 1999; Sweet *et al.*, 1999. McClay *et al.*, 2000) and enhance endoderm specification (Ransick and Davidson, 1995; Logan *et al.*, 1999). The requirement of the micromeres for LvBrac expression was therefore assayed by removing micromeres from 16-cell embryos and later staining these embryos for LvBrac. Remarkably, the torus of LvBrac appeared at the normal time and position in all such manipulated embryos (Fig. 8A). This indicates that micromere induction is not necessary for LvBrac expression in this region.

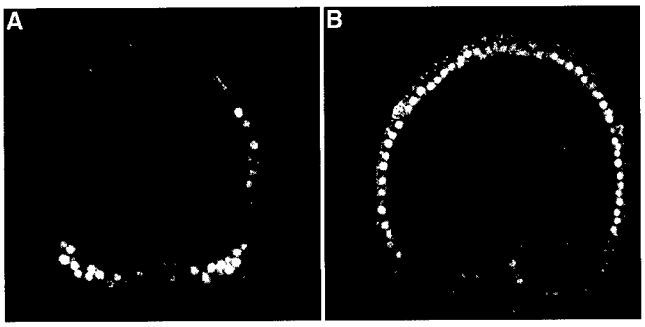

FIG. 6. $NiCl_2$ treatment and localization of LvBrac. (A) Normal LvBrac expression. (B) Expression of LvBrac after exposure to 1 mM $NiCl_2$. LvBrac is now expressed in the entire ectodermal region of the embryo.

Since LvBrac is expressed in veg1 endoderm later than in veg2 endoderm, it was then of interest to determine whether the veg1 LvBrac expression was also autonomous as in the veg2 derivatives or if an inductive signal from the underlying veg2 layer was necessary for proper LvBrac expression in veg1. Such a veg2–veg1 inductive signal has been shown to be necessary for proper veg1 regional cell identity within the archenteron (Logan and McClay, 1999). To determine the autonomy of LvBrac expression in veg1, micromeres were removed at the 16-cell stage as above and the veg2 layer was subsequently removed at the 60-cell stage when the veg2 and veg1 layers first arise. LvBrac staining was observed in the remaining veg1-derived cells when assayed at 16 h postfertilization, the time at which control embryos had reached midgastrula stage (Fig. 8B). This expression is in a vegetal patch rather than a torus, likely due to the removal of all of the veg2-derived mesoderm and endoderm normally present in the center of the plate thereby apposing the adjacent veg1-derived endoderm cells. Thus, the veg1 LvBrac expression is not dependent on the veg2 inductive signal.

β-Catenin has been demonstrated to be necessary for endoderm specification (Wikramanayake *et al.*, 1998; Logan *et al.*, 1999). Nuclear β-catenin is present in the micromere and macromere cell tiers at 5th cleavage. Between 6th and 7th cleavages, however, nuclear β-catenin protein remains in veg2 nuclei while protein levels are significantly lower in veg1 nuclei (Logan *et al.*, 1999). Later, another dynamic change in β-catenin expression occurs prior to the onset of gastrulation where veg1 nuclei destined to become

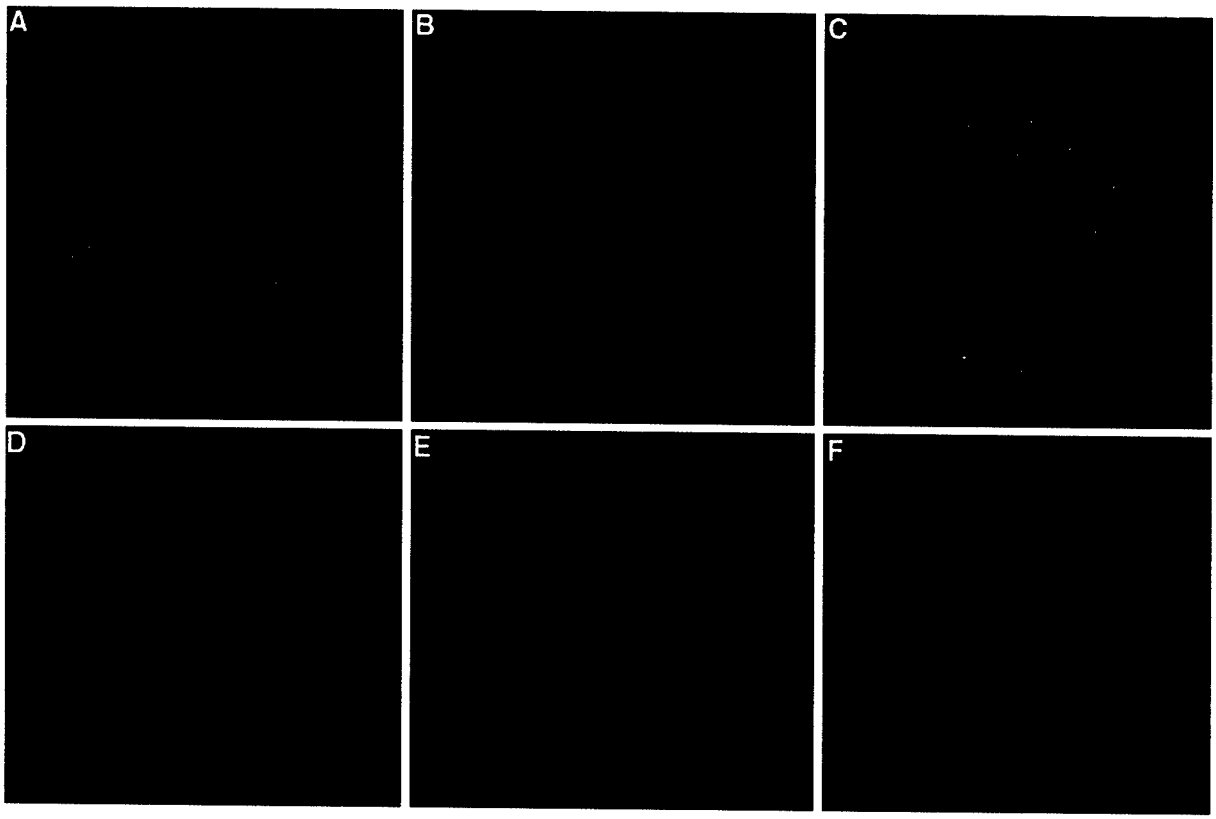

FIG. 7. Colocalization of LvBrac with mesodermal and endo/mesodermal markers. (A) Early gastrula embryo doubled labeled with the PMC marker, 1g8 (green) and LvBrac (red) showing that LvBrac is not expressed in PMCs. (B-E) Embryos double labeled an antibody that recognizes the extracellular domain of LvNotch (green) and LvBrac (red). In mesenchyme blastula stages, LvNotch is absent in the presumptive SMCs central in the vegetal plate and is upregulated apically at the endoderm side of the endoderm-mesoderm boundary. LvBrac does not localize to SMCs when viewed from the surface (C) or in cross section (B, D, E) Cross section of early gastrula (D) and late gastrula (E) embryos labeled as in (B, C). LvBrac does not localize to any mesodermal tissues (PMCs or SMCs) and additionally, remains in a static circumference relative to the invaginating endoderm cells marked by apical LvNotch passing through that circumference. (F) mRNA injection of LvNotch^{act} results in an increase in SMC cell types. Animal view of representative embryo stained with LvBrac. Again, no nuclei in the ectopic SMCs stain positive for LvBrac.

endoderm again express high levels of nuclear β-catenin relative to those destined to become ectoderm within that tier. Micromereless embryos retain the temporal and spatial expression pattern of β-catenin, indicating that the micromere signal is not necessary for proper expression in the prospective endoderm (Logan *et al.*, 1999). In both veg2 and veg1 nuclei, LvBrac expression follows, with a temporal delay, expression of nuclear β-catenin; and as with β-catenin, LvBrac is autonomously expressed (Figs. 8A and 8B). It was therefore determined whether perturbations to Wnt/β-catenin signaling resulted in alterations in the expression pattern of LvBrac. Embryos were treated with 50 mM LiCl and subsequently stained for LvBrac expression. LiCl is thought to exert its vegetalizing effect by inhibiting GSK3-β (Klein and Melton, 1996), thereby stabilizing β-catenin, and thus increasing the amount of endoderm and mesoderm at the expense of ectoderm in the embryo (Livingston and Wilt, 1989; Emily-Fenouil *et al.*, 1998). Embryos treated in this fashion exhibit increased LvBrac expression at mesenchyme blastula stage relative to controls (Fig. 8C, control mesenchyme blastula staining identical to Figs. 4A and 4B). Expression is maintained in a torus around the blastopore; the torus, however, is positioned higher along the animal–vegetal axis, reflecting the increase in mesoderm at the center of the vegetal plate. The converse manipulation, that of preventing β-catenin from gaining access to the nucleus and thereby preventing activation of downstream genes, can be achieved by ectopically expressing the cytoplasmic tail of E-cadherin serving as a "sink" to bind all free β-catenin. Injection of mRNA encoding the cytoplasmic tail of the sea urchin E-cadherin orthologue, LvG-cadherin (Miller and McClay, 1997), results in an animalized phenotype where embryos lack all endoderm and mesoderm (Logan *et al.*, 1999). Such embryos also lack LvBrac expression (Fig. 8D).

Functional Characterization of LvBrac

The striking expression pattern of LvBrac around the blastopore suggested the possibility that it is necessary

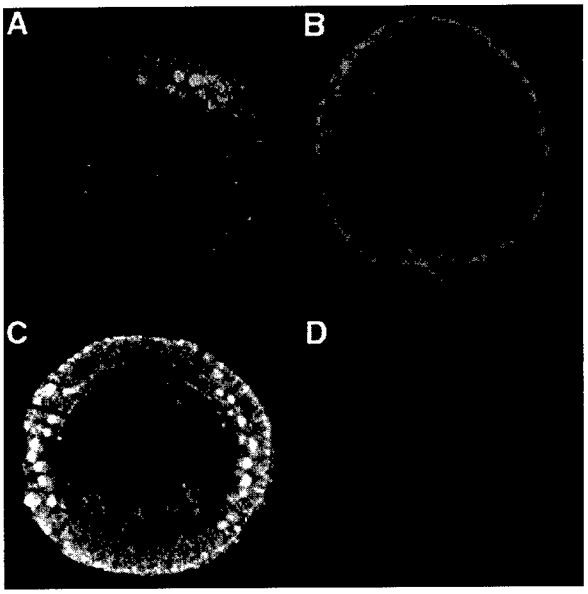

FIG. 8. Vegetal LvBrac expression is not dependent upon micromere or micromere/veg2 inductive signals and is altered in response to perturbations to β-catenin. (A) Micromeres were eliminated from 16-cell stage embryos, an operation that prevents induction of SMCs, delays gastrulation and prevents enhancement of endoderm marker gene expression. At early gastrula stages (12–13 h, verified in control sibling embryos), micromereless embryos were stained with antibodies to LvBrac. Vegetal LvBrac expression is unaffected as it appears in a spatially and temporally normal fashion. (B) Embryos from which the micromeres and veg2 cell layer were eliminated. Vegetal LvBrac expression is present at mid gastrula stages (16 h, verified in control sibling embryos). (C) Treatment of embryos at the 8 cell stage with 50 mM LiCl results in an overproduction of endoderm and mesoderm at the expense of ectoderm. Such embryos express increased LvBrac levels. (D) Injection of ΔLvG-cadherin mRNA animalizes the embryo through binding to endogenous β-catenin and preventing nuclear localization. These embryos do not express LvBrac protein.

during gastrulation for the involution of endoderm tissues. To test this, a construct was engineered analogous to that employed in *Xenopus* (Conlon *et al.*, 1996). The DNA binding domain of LvBrac was fused to the transcriptional repression domain of the *Drosophila* engrailed gene thereby making the resulting hybrid construct a transcriptional repressor (LvBrac-EN). Microinjection of LvBrac-EN mRNA into fertilized sea urchin eggs resulted in highly consistent phenotypes (Fig. 9). Injection of glycerol, EN, or low levels of LvBrac-EN mRNA (≤0.19 pg/pl) had no effect as phenotypically normal embryos resulted when assayed at late gastrula stages (Fig. 9A). Medium level LvBrac-EN mRNA injection (0.375 pg/pl) resulted in three phenotypes, normal embryos (18%), a delayed gastrulation phenotype where some embryos had archenterons roughly half the length of their control siblings at late gastrula stage (38%, data not shown), or a complete block in gastrulation (44%; Fig. 9B). Higher level LvBrac-EN mRNA injection (>0.375 pg/pl) resulted in a block to gastrulation in all embryos. A small amount of involuted tissue occurred in many of these nongastrulating embryos, but in none did the tissue traverse more than one quarter the length of the blastocoel.

Next, it was determined whether LvBrac-EN mRNA injection perturbed endoderm or mesoderm gene expression. PMCs were present in all LvBrac-EN-injected embryos (Fig. 9B) and these injected embryos stained positively for the PMC marker 1g8 (data not shown). In some cases of higher quantity mRNA injections (≥1 pg/pl), it appeared that not all PMCs had ingressed from the vegetal plate. Overexpression at this level perhaps was toxic or interfered with another T-box gene present in the PMCs. LvBrac-EN-injected embryos also expressed normal levels of apical LvNotch (Fig. 9C) and stained positively for SMC-1 (Fig. 9D), indicating a proper endoderm-secondary mesoderm boundary was formed. Thus, it is likely that LvBrac is not integral for the specification of endoderm but is necessary for its morphogenesis.

LvBrac-EN-injected embryos were also observed at 48 h postfertilization (hpf) to score gut and mouth formation. Control glycerol-injected embryos are depicted in Figs. 9E and 9G. Embryos at this stage possess a full skeleton, tripartite gut (Fig. 9E), and stomodaeum (Fig. 9G). Nearly all LvBrac-EN embryos injected with <1 pg/pl mRNA appeared identical to controls at this stage (data not shown), indicating that after the LvBrac-EN mRNA is degraded, the embryos regulate and recover from the LvBrac-EN perturbation. When injected with high amounts of LvBrac-EN mRNA (≥1 pg/pl) and assayed at 48 hpf, most embryos also looked identical to controls. Some embryos, at a low frequency, retained defects in the endoderm within this high LvBrac-EN mRNA group (Figs. 9F and 9H).

The second defect observed in high (≥1 pg/pl) LvBrac-EN embryos was the lack of a stomodaeum (Fig. 9H). This phenotype was very rare but was the only other defect noticed in more than 150 injected embryos scored at 48 hpf. The low level of persistent defects at 48 hpf likely relates to the lack of perdurance of the LvBrac-EN mRNA and LvBrac-EN protein in the embryo prior to stomodaeum invagination, as well as the extensive regulative capacity of this embryo. Studies on expressed LvNotch mRNA constructs and their protein products indicate that expression peaks at 8–12 h (blastula stage) and perdures into gastrula stages (Sherwood and McClay, 1999). As well, the capacity for regulative changes in the gut continues beyond this time (McClay and Logan, 1996). Both defects in this low frequency of embryos, however, relate to sites of LvBrac expression and prevent normal morphogenesis in those regions.

To test the autonomy of LvBrac-EN effects, mRNA injections were performed at the two-cell stage. One of two blastomeres was injected with LvBrac-EN in a solution containing FITC-dextran in order to follow the injected half of the resulting embryo. Surprisingly, when one blastomere was injected with LvBrac-EN, a normal embryo resulted

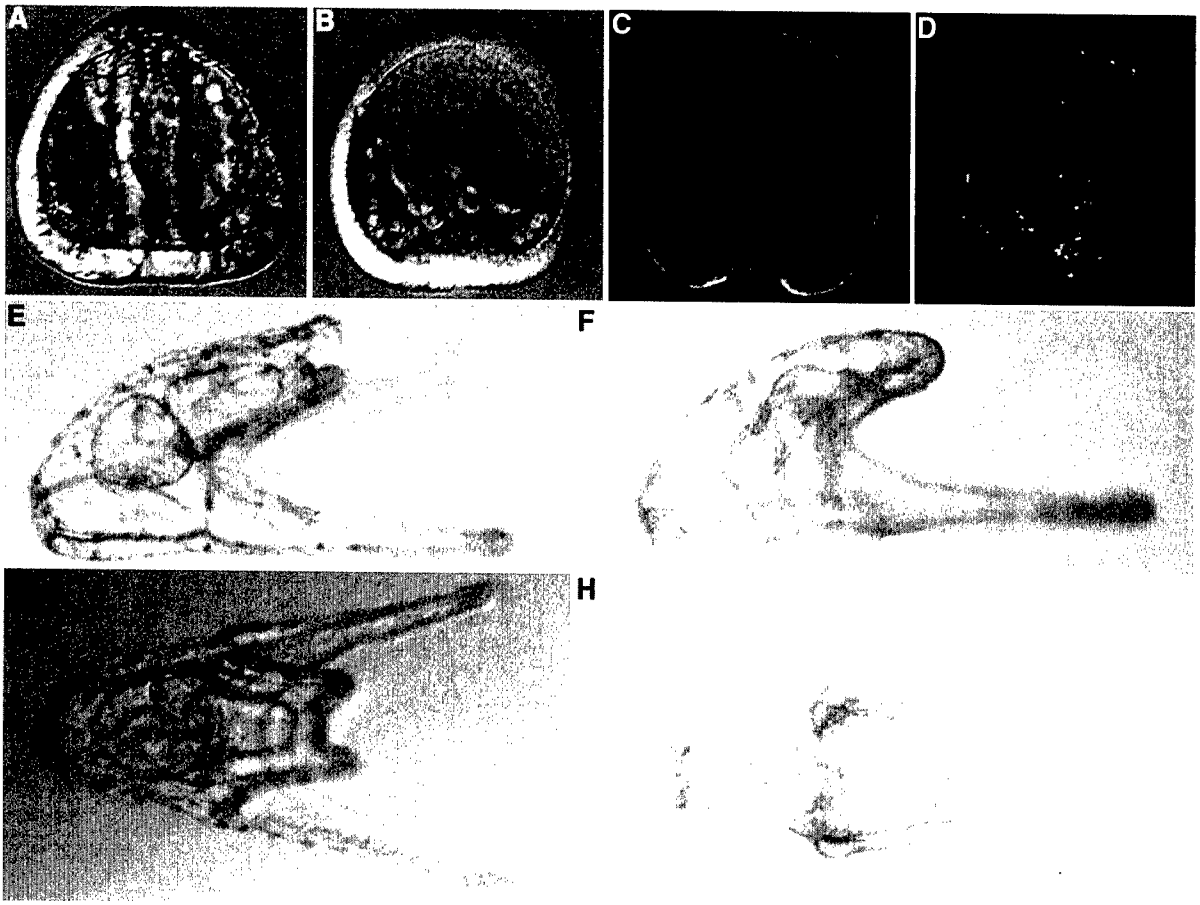

FIG. 9. Perturbation to LvBrac function via microinjection of mRNA encoding a LvBrac/*Drosophila*-Engrailed protein. Nomarski images and endomesodermal marker staining of control and LvBrac-EN expressing embryos. (A) Appearance of control embryos injected with glycerol (22 hpf). (B) Phenotype of LvBrac-EN injected embryo (22hpf). Note the presence of migrating PMCs (arrowhead) and the smaller gut invagination relative to the control embryo depicted in (a). (C, D) Apical LvNotch expression (C) and SMC-1 expression (oblique cross section, D) in LvBrac-EN injected embryo indicating that endoderm and secondary mesoderm are present. (E-H) Nomarski images of 48 hpf embryos. (E) Control glycerol injected embryo with normal tripartite gut. (F) High level LvBrac-EN mRNA injected embryo. One defect in the high LvBrac-EN mRNA injected group was the occurrence of embryos with smaller guts compared to those of controls at a low frequency. These embryos possessed tripartite guts but these guts were clearly composed of less tissue. Additionally, these embryos often possessed fewer pigment cells when compared to control glycerol injected siblings. As LvBrac-EN injected embryos stain positively for SMC-1 (D) and possess other mesodermal cell types at 48 hpf (coelomic sacs and blastocoelar cells, data not shown), this could reflect the block to PMC ingression (see text) from high level ($\geq$ 1 pg/pl) LvBrac-EN mRNA injection and subsequent regulative conversion of pigment cells to PMCs to form a proper skeleton (Ettensohn and Ruffins 1993). (G) Stomodaeum formation in control glycerol injected embryo. (H) High level LvBrac-EN injected embryo lacking a stomodaeum.

with an archenteron composed of progeny from both the injected and uninjected blastomeres, similar to that observed in control glycerol injections (Fig. 10). Apparently, the progeny of the uninjected blastomere, being capable of normal gastrulation movements, are able to influence the progeny of the LvBrac-EN-injected blastomere so that these cells invaginate along with control cells into the blastocoel. The experiment does not discriminate between either the possibility that the control cells can somehow mechanically pull the LvBrac-EN-injected cells into the archenteron or the possibility that the control cells provide a necessary signal that rescues the ability of the LvBrac-EN-injected cells to invaginate into the blastocoel.

DISCUSSION

LvBrac Is Expressed in Distinct Animal and Vegetal Regions of the Sea Urchin Embryo

LvBrac expression first appears at mesenchyme blastula stage in two distinct regions. In the animal hemisphere, expression appears in a large patch of cells in the oral

ectoderm. More nuclei of the oral field initially express LvBrac than the number of cells comprising the stomodaeum. Thus, a broad expression is later refined to remain only in the cells of the mouth. Expression continues in the stomodaeum into the pluteus stage. Vegetally, LvBrac protein first appears circumferentially around the blastopore and continues to be expressed there through gastrula stages, surrounding the anus into the pluteus stage.

In situ expression patterns in the urchin for HpTa and SpBra (Harada *et al.*, 1995; Peterson *et al.*, 1999) differ somewhat from that reported here for LvBrac protein. Pan-vegetal plate (endoderm, PMC, and SMC) localized mRNA expression was reported in these species with later refinement to the SMCs at the tip of the archenteron during gastrulation. In the present study, however, at the protein level, LvBrac was never observed in PMCs or SMCs even when excess SMCs were produced via LvN^{act} mRNA injection. Additionally, LvBrac protein is clearly expressed in the oral ectoderm and stomodaeum, expression not observed in the *in situ* patterns for HpTa or SpBra but consistent with that observed for *Brachyury* mRNA in both starfish and hemichordate embryos (Tagawa *et al.*, 1998; Shoguchi *et al.*, 1999).

Dynamic Expression and Function of LvBrac

That LvBrac is expressed dynamically in an endoderm ring around the blastopore has several implications as to its regulation and function. LvBrac protein must be rapidly synthesized then degraded since cells move into and then exit the ring of LvBrac expression as they move into the blastopore. Upon involution, expression in those cells ceases and protein is rapidly degraded, while in adjacent noninvoluted cells expression begins. This dynamic wave of expression continues until all endoderm tissue has involuted into the blastocoel, at which point, expression is maintained in the anal region of the hindgut. Thus, vegetal LvBrac expression is exclusively endodermal, initiating at the endoderm–mesoderm boundary and terminating at the endoderm–ectoderm boundary.

Perturbation of LvBrac function in the sea urchin suggests that it is necessary for endodermal movements during gastrulation. The perturbed cells express an endoderm boundary marker in a temporally normal manner. It appears likely that LvBrac perturbation blocks the endoderm's ability to invaginate rather than its specification although more specific endoderm marker staining in LvBrac-EN-injected embryos is necessary to conclusively demonstrate this. Phenotypes of LvBrac-EN-injected embryos are similar to described mutants in mouse and zebrafish (Chelsey, 1935; Kimmel *et al.*, 1989) and functional perturbation in *Xenopus* (Conlon *et al.*, 1996; Conlon and Smith, 1999). In each of these cases, phenotypes most likely manifest due to defective gastrulation movements.

The remnant of invagination seen in LvBrac-EN-injected embryos likely corresponds to early ingressing SMC cell types (Fig. 9D; Ruffins and Ettensohn, 1996). Since these cells do not express LvBrac, their normal morphogenetic movement in the presence of LvBrac-EN indicates that they are unaffected by modifications to the endoderm and are therefore free to invaginate. Further archenteron involution is blocked, however, due to LvBrac-EN perturbation of the adjacent endodermal cells.

The removal of micromeres has little effect on LvBrac expression. Micromere removal blocks SMC specification while endoderm is known to be specified largely autonomously (Logan *et al.*, 1999) with micromere signaling participating in elevating the levels of endoderm marker expression (Ransick and Davidson, 1995). That inductive interaction is not required for LvBrac expression and therefore the expression of LvBrac in the endoderm of micromereless embryos suggests that it is synthesized as a consequence of the autonomous endoderm specification sequence involving β-catenin. Gastrulation, however, is delayed in micromereless embryos likely due to the lack of the enhancement of endodermal gene expression provided by the micromeres. Removal of micromeres and the veg2 cell layer results in the formation of a small gut derived from veg1 cells (Horstadius, 1973). This gut does not extend to the oral ectoderm and expresses only midgut and hindgut markers (Khaner and Wilt, 1991; Logan and McClay, 1999). If the veg1 cells are induced by the veg2 layer below them, they will differentiate into a greater range of gut tissues (Logan and McClay, 1999). Nevertheless, this inductive signal is also not necessary for LvBrac expression in veg1. Therefore, LvBrac expression in endoderm depends primarily on initial autonomous endoderm specification events in macromeres.

Upstream of LvBrac

The noncontiguous animal and vegetal expression domains of LvBrac are most likely indicative of distinct *cis*-regulatory modules controlling the spatial and temporal expression in these regions (Davidson, 1993, 1998). One obvious candidate for regulating endodermal LvBrac expression is β-catenin or genes downstream of β-catenin/TCF-Lef signaling. Elimination or augmentation of that pathway correspondingly alters LvBrac expression. Identification and characterization of the LvBrac promoter region as well as screens directed at identifying targets of β-catenin/Wnt signaling will further elucidate these observations and determine whether LvBrac is a direct target or is expressed in response to a progression of endodermal specification.

Downstream Targets of Brachyury

Injection of LvBrac-EN into one of two blastomeres results in the injected side invaginating normally while in embryos injected at the one-cell stage, invagination is blocked. This observation is curious in that it suggests possible nonautonomy in LvBrac downstream function. Screens for targets of *Brachyury* in *Ciona intestinalis* and *Xenopus laevis* have been recently reported (Tada *et al.*, 1998; Takahashi *et al.*, 1999; Saka *et al.*, 2000). Genes were

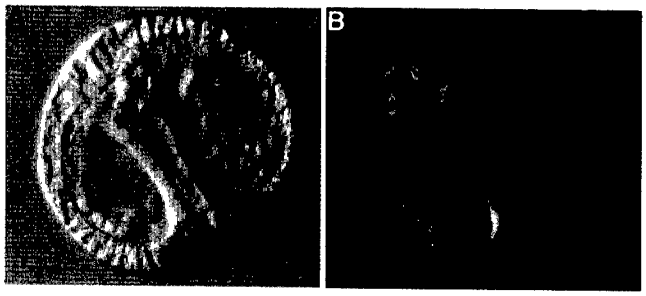

FIG. 10. (A) Nomarski image of a late gastrula stage embryo resulting from injection of LvBrac-EN mRNA into one blastomere at the 2-cell stage. In these embryos, gastrulation occurs normally. (B) Fluorescence signal arising from progeny of the LvBrac-EN injected blastomere. Note that the archenteron is composed of progeny from both injected and uninjected blastomeres.

identified in these screens homologous to extracellular matrix components, cytoskeletal proteins, adhesion molecules, membrane receptors, nuclear proteins, and signaling proteins, indicating that *Brachyury* induces the expression of a variety of genes involved in diverse cellular processes. Two genes of interest identified in *Xenopus* as targets of Xbra are FGF and Xwnt11 (Schulte-Merker and Smith, 1995; Casey *et al.*, 1998; Saka *et al.*, 2000). Expression of eFGF is similar to that of Xbra and the two are thought to participate in an autoregulatory loop where Xbra activates eFGF expression, which in turn maintains the expression of Xbra (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). Expression of Xwnt11 is dependent on *Brachyury* and parallels that of Xbra at gastrula and neurula stages (Saka *et al.*, 2000). Overexpression of a dominant negative form of Xwnt11, like expression of Xbra-EN, inhibits convergent extension movements in *Xenopus* (Tada and Smith, 2000). A similar phenotype is observed in the ENU-induced zebrafish Silberblick mutant (Heisenberg *et al.*, 2000). The mechanism whereby theseWnt11proteins function appears to be novel in that they act through a noncanonical Wnt pathway and resemble signaling observed in *Drosophila* planar polarity (reviewed in Adler, 1992; Boutros and Mlodzik, 1999). If such a relationship exists between *Brachyury* expression and subsequent downstream FGF or Wnt expression for gastrulation movements in the sea urchin, one would predict that LvBrac would function nonautonomously in coordinating these cell movements.

Evolutionary Changes in Brachyury Expression and Function

LvBrac expression is maintained in the stomodaeum and anal region of the pluteus hindgut. By comparison, *Brachyury* orthologues in starfish and hemichordates display both stomodael and hindgut mRNA expression patterns (Tagawa *et al.*, 1998; Shoguchi *et al.*, 1999) while *Drosophila* and *C. elegans* Brachyury orthologues, *brachyenteron* and *mab-9*, and the Larvacean urochordate (*Oikopleura dioica*) *Brachyury* orthologue are all expressed in the developing hindgut (Singer *et al.*, 1996; Woollard and Hodgkin, 2000; Bassham and Postlethwait, 2000). These data support an ancient role for *Brachyury* in the stomodaeum and posterior hindgut of the protostome/deuterostome ancestor (Peterson *et al.*, 1999b). Upon divergence of protostomes and deuterostomes, *Brachyury* utilization further evolved. In indirect developing protostomes (trochophore primary larva; *Platynereis*), expression was maintained in this fashion (Arendt *et al.*, 2001), while in the direct developing protostomes (nontrochophore primary larva; *Drosophila* and *C. elegans*) stomodael expression was lost (Singer *et al.*, 1996; Woollard and Hodgkin, 2000). In the nonchordate deuterstome lineages (echinoderms and hemichordates), embryonic expression and function was maintained in the stomodaeum and hindgut. Here, similar morphogenetic mechanisms of invagination are present and therefore, perhaps the ancestral role of *Brachyury* is in invagination processes. In the chordate lineages (urochordates, cephalochordates, and vertebrates), expression and function was lost in the stomodaeum but expanded into the mesoderm, and particularly the notochord. Additionally, in members of at least some of the chordate lineages (urochordates and vertebrates), *Brachyury* expression was maintained in the hindgut endoderm (Bassham and Postlethwait, 2000; Gont *et al.*, 1993). For example, in addition to Larvacean urochordates, in the *Xenopus* embryo, Xbra mRNA is expressed in the posterior or postanal gut stretching from the anus to the tailbud (Gont *et al.*, 1993). *Brachyury* expression and function is therefore consistent with a role in invagination processes and stomodael/posterior endoderm formation in both protostomes and deuterstomes. In indirect developing protostomes and some deuterstomes, this expression and function is maintained in some lineages and lost in others, while new roles for *Brachyury* appear to have evolved. In all chordate lineages, a novel role in mesoderm formation appeared. Additional SpBra expression in the sea urchin appears later during larval phases, *in situ*, in the mesodermal hydrocoel and the vestibule (Peterson *et al.*, 1999a). It will be interesting to characterize larval expression prior to, and during metamorphosis as this is a period of incredible morphogenetic movements.

ACKNOWLEDGMENTS

We thank Drs. Lynne Angerer and Sergei Sokol for supplying constructs. We would like to thank Dr. Charles Ettensohn, Dr. John Klingensmith and both past and present members of the McClay lab for suggestions and discussions during the course of this work. Particularly we would like to thank Drs. David Sherwood and Robert Peterson for extensive discussion of the studies herein and critical comments on this manuscript, Dr. Michael Ferkowicz for ΔLvG-cadherin injected embryos, Kirk Zigler for help with phylogenetics and John Cash for technical assistance. A portion of this study was performed at the Bermuda Biological

Station for Research and we are grateful to Michelle Rau for assistance while there. This work was supported by NIH grants HD14483 and GM64164 to D.R.M. and Department of Defense Breast Cancer Research Program grant BC990657 to J.M.G.

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Received for publication July 17, 2001
Revised August 14, 2001
Accepted August 14, 2001
Published online September 28, 2001